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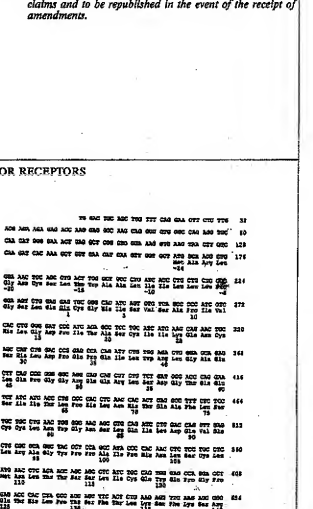
(54) Title: GRANULOCYTE-COLONY STIMULATING FACTOR RECEPTORS

(57) Abstract

Mammalian granulocyte-colony stimulating factor (G-CSF) receptor proteins, DNAs and expression vectors encoding mammalian G-CSF receptors, and processes for producing mammalian G-CSF receptors as products of recombinant cell culture, are disclosed.



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TITLE

Granulocyte-Colony Stimulating Factor Receptors

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of U.S. Application Serial No. 522,952, filed April 3, 1990, which is a continuation-in-part of U.S. Application Serial No. 416,306, filed October 3, 1989, which is a continuation-in-part of U.S. Application Serial No. 412,816, filed on September 26, 1989.

BACKGROUND OF THE INVENTION

The present invention relates generally to cytokine receptors and more specifically to granulocyte-colony stimulating factor receptors.

Human Granulocyte-Colony Stimulating Factor (G-CSF) is a lineage-specific hematopoietic protein which stimulates the proliferation and differentiation of granulocyte-committed progenitor cells. Human G-CSF has also been shown to functionally activate mature neutrophils. The cDNAs for human (Nagata et al., *Nature 319*;415, 1986) and mouse G-CSF (Tsuchiya et al., *PNAS* 83, 7633, 1986) have been isolated, permitting further structural and biological characterization of G-CSF.

G-CSF initiates its biological effect on cells by binding to specific G-CSF receptor protein expressed on the plasma membrane of a G-CSF responsive cell. Because of the ability of G-CSF to specifically bind G-CSF receptor (G-CSFR), purified G-CSFR compositions will be useful in diagnostic assays for G-CSF, as well as in raising antibodies to G-CSF receptor for use in diagnosis and therapy. In addition, purified G-CSF receptor compositions may be used directly in therapy to bind or scavenge G-CSF, thereby providing a means for regulating the immune activities of this cytokine. In order to study the structural and biological characteristics of G-CSFR and the role played by G-CSFR in the responses of various cell populations to G-CSF or other cytokine stimulation, or to use G-CSFR effectively in therapy, diagnosis, or assay, purified compositions of G-CSFR are needed. Such compositions, however, are obtainable in practical yields only by cloning and expressing genes encoding the receptors using recombinant DNA technology. Efforts to purify the G-CSFR molecule for use in biochemical analysis or to clone and express mammalian genes encoding G-CSFR have been impeded by lack of a suitable source of receptor protein or mRNA. Prior to the present invention, no cell lines were known to express high levels of G-CSFR constitutively and continuously, which precluded purification of receptor for sequencing or construction of genetic libraries for direct expression cloning.

SUMMARY OF THE INVENTION

The present invention provides DNA sequences encoding mammalian granulocyte-colony stimulating factor receptors (G-CSFR) or subunits thereof. Preferably, such DNA sequences are selected from the group consisting of (a) cDNA clones having a nucleotide sequence derived from the coding region of a native G-CSFR gene; (b) DNA sequences which are capable of hybridization to the cDNA clones of (a) under moderately stringent conditions and which encode biologically active G-CSFR molecules; and (c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode biologically active G-CSFR molecules. The present invention also provides recombinant expression vectors comprising the DNA sequences defined above, recombinant G-CSFR molecules produced using the recombinant expression vectors, and processes for producing the recombinant G-CSFR molecules using the expression vectors.

The present invention also provides isolated or purified protein compositions comprising mammalian G-CSFR. Preferred G-CSFR proteins are soluble forms of the native receptors.

The present invention also provides compositions for use in therapy, diagnosis, assay of G-CSFR, or in raising antibodies to G-CSFR, comprising effective quantities of soluble native or recombinant receptor proteins prepared according to the foregoing processes. These and other aspects of the present invention will become evident upon reference to the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows restrictions maps of cDNA clones D-7 and 25-1 containing regions encoding human G-CSFR proteins.

FIGURES 2-5 depict that cDNA sequence of clone D-7 which was isolated from a human placental library, and the predicted amino acid sequence of this clone. The coding region of the predicted mature full-length membrane-bound protein from clone D7 is defined by amino acids 1-759. The predicted N-terminal Glu of the mature protein is designated amino acid number 1 and is underlined. The putative transmembrane region at amino acids 604-629 is also underlined.

FIGURE 6 depicts the 3' nucleotide sequence and predicted C-terminal amino acid sequence of clone 25-1, which is the result of an alternative splicing arrangement. The position of the intron insertion in clone 25-1 is indicated with a ↓ after nucleotide 2411 of Figure 1. The position of the intron-exon boundaries are indicated with a ↓, and splice-donor and splice-acceptor recognition sequences are boxed. Sequences also present in clone D-7 are underlined.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

G-CSF is a growth factor which induces growth and differentiation of neutrophilic granulocyte progenitors. The biological activities of G-CSF are mediated through binding to specific cell surface receptors, referred to as "G-CSF receptors" or "G-CSFR". G-CSFR, as used herein, refers to proteins having amino acid sequences which are substantially similar to native mammalian G-CSFR amino acid sequences, such as the human G-CSFR sequence disclosed in Figure 1, or fragments thereof, and which are biologically active as defined below, in that they are capable of binding G-CSF molecules or, in their native configuration as intact human plasma membrane proteins, transducing a biological signal initiated by a G-CSF molecule binding to a cell, or cross-reacting with anti-G-CSFR antibodies raised against G-CSFR from natural (i.e., nonrecombinant) sources. Specific embodiments of G-CSFR include polypeptides substantially equivalent to the sequence of amino acids 1-759 of Figures 2-5 (clone D-7) or the sequence of amino acids 1-776 of the protein encoded by clone 25-1 as disclosed in Figures 2-5 and 6. The terms "G-CSF receptor" or "G-CSFR" include, but are not limited to, soluble G-CSF receptors, as defined below. As used throughout this specification, the term "mature" means a protein expressed in a form lacking a leader sequence as may be present in full-length transcripts of a native gene. Various bioequivalent protein and amino acid analogs are described in detail below.

The mature N-terminal amino acid is predicted to be Glu^{1} (underlined and designated as amino acid 1 in Figures 2-5), based on the algorithm of von Heijne, G., *Nucl. Acids Res. 14*:4683 (1986), for determining signal cleavage sites. However, several factors suggest that Ser^{-3} may be the correct mature N-terminal amino acid, based on the observation that Ser^{-3} is 21 amino acids from the N-terminal Met and is preceded by the small amino acid residue Gly, both of which are accepted criteria for identifying signal cleavage sites. The actual N-terminal amino acid of the mature protein can be confirmed by sequencing purified G-CSFR protein using standard techniques. Thus, amino acid sequences equivalent to those described above include, for example, amino acids -3 through 759 of Figures 2-5 (clone D-7) or -3 through 776 of the protein encoded by clone 25-1 as disclosed in Figures 2-5 and 6.

In their native configuration, receptor proteins are present as intact human plasma membrane proteins having an extracellular region which binds to a ligand, a hydrophobic transmembrane region which causes the protein to be immobilized within the plasma membrane lipic bilayer, and a cytoplasmic or intracellular region which interacts with cytoplasmic proteins and/or chemicals to deliver a biological signal to effector cells via a cascade of chemical reactions within the cytoplasm of the cell. The hydrophobic

transmembrane region and a highly charged sequence of amino acids in the cytoplasmic region immediately following the transmembrane region cooperatively function to halt transport of the G-CSFR across the plasma membrane. "Soluble G-CSFR" or sG-CSFR", as used in the context of the present invention, refer to a protein, or a substantially equivalent analog, having an amino acid sequence corresponding to the extracellular region of native G-CSFR, for example polypeptides having the amino acid sequences substantially equivalent to the sequences of amino acids 1-603 of Figures 2-5. Equivalent sG-CSFRs include polypeptides which vary from the sequences shown in Figures 2-5 by one or more substitutions, deletions, or additions, and which retain the ability to bind G-CSF and inhibit the ability of G-CSF to transduce a signal via cell surface bound G-CSF receptor proteins. Because sG-CSFR proteins are devoid of a transmembrane region, they are secreted from the host cell in which they are produced. Equivalent soluble G-CSFR include, for example, the sequence of amino acids -3 through 603 of Figures 2-5. When administered in therapeutic formulations, sG-CSFR proteins circulate in the body and bind to circulating G-CSF molecules, preventing interaction of G-CSF with natural G-CSF receptors and inhibiting transduction of G-CSF-mediated biological signals, such as immune or inflammatory responses. The ability of a polypeptide to inhibit G-CSF signal transduction can be determined by transfecting cells with recombinant G-CSF receptor DNAs to obtain recombinant receptor expression. The cells are then contacted with G-CSF and the resulting metabolic effects examined. If an effect results which is attributable to the action of the ligand, then the recombinant receptor has signal transducing activity. Examplary procedures for determining whether a polypeptide has signal transducing activity are disclosed by Idzerda et al., *J. Exp. Med.* 171:861 (1990); Curtis et al., *Proc. Natl. Acad. Sci. USA 86*:3045 (1989); Prywes et al., *EMBO J. 5*:2179 (1986); and Chou et al., *J. Biol. Chem. 262*:1842 (1987). Alternatively, primary cells of cell lines which express an endogenous G-CSF receptor and have a detectable biological response to G-CSF could also be utilized.

"Substantially similar" G-CSFR include those whose amino acid or nucleic acid sequences vary from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which is to retain biological activity of the G-CSFR protein. Alternatively, nucleic acid subunits and analogs are "substantially similar" to the specific DNA sequences disclosed herein if: (a) the DNA sequence is derived from the coding region of a native mammalian G-CSFR gene; (b) the DNA sequence is capable of hybridization to DNA sequences of (a) under moderately stringent conditions and which encode biologically active G-CSFR molecules; or DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) or (b) and which encode biologically active G-CSFR molecules. Substantially similar analog proteins will be greater

than about 30 percent similar to the corresponding sequence of the native G-CSFR. Sequences having lesser degrees of similarity but comparable biological activity are considered to be equivalents. More preferably, the analog proteins will be greater than about 80 percent similar to the corresponding sequence of the native G-CSFR, in which case they are defined as being "substantially identical." In defining nucleic acid sequences, all subject nucleic acid sequences capable of encoding substantially similar amino acid sequences are considered substantially similar to a reference nucleic acid sequence. Percent similarity may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol. 48*:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math. 2*:482, 1981). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res. 14*:6745, 1986, as described by Schwartz and Dayhoff, ed., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

"Recombinant," as used herein, means that a protein is derived from recombinant (e.g., microbial or mammalian) expression systems. "Microbial" refers to recombinant proteins made in bacterial or fungal (e.g., yeast) expression systems. As a product, "recombinant microbial" defines a protein produced in a microbial expression system which is essentially free of native endogenous substances. Protein expressed in most bacterial cultures, e.g., *E. coli*, will be free of glycan. Protein expressed in yeast may have a glycosylation pattern different from that expressed in mammalian cells.

"Biologically active," as used throughout the specification as a characteristic of G-CSF receptors, means that a particular molecule shares sufficient amino acid sequence similarity with the embodiments of the present invention disclosed herein to be capable of binding detectable quantities of G-CSF, transmitting a G-CSF stimulus to a cell, for example, as a component of a hybrid receptor construct, or cross-reacting with anti-G-CSFR antibodies raised against G-CSFR from natural (i.e., nonrecombinant) sources. Preferably, biologically active G-CSF receptors within the scope of the present invention are capable of binding greater than 0.1 nmoles G-CSF per nmole receptor, and most

preferably, greater than 0.5 nmole G-CSF per nmole receptor in standard binding assays (see below).

"DNA sequence" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct, which has been derived from DNA isolated at least once in substantially pure form, i.e., free of contaminating endogenous materials and in a quantity or concentration enabling identification, manipulation, and recovery of the sequence and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. Genomic DNA containing the relevant sequences could also be used. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

"Nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides. DNA sequences encoding the proteins provided by this invention can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being expressed in a recombinant transcriptional unit.

"Recombinant expression vector" refers to a replicable DNA construct used either to amplify or to express DNA which encodes G-CSFR and which includes a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences. Structural elements intended for use in yeast expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

"Recombinant microbial expression system" means a substantially homogeneous monoculture of suitable host microorganisms, for example, bacteria such as *E. coli* or yeast such as *S. cerevisiae*, which have stably integrated a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit as a component of a resident plasmid. Generally, cells constituting the system are the progeny of a single ancestral transformant. Recombinant expression systems as defined herein will express heterologous protein upon induction of the regulatory elements linked to the DNA sequence or synthetic gene to be expressed.

The term "isolated", as used in the context of this specification to define the purity of a G-CSFR or sG-CSFR protein or protein composition, means that the protein or protein composition is substantially free of other proteins of natural or endogenous origin and contains less than about 1% by mass of protein contaminants residual of production processes. Such compositions, however, can contain other proteins added as stabilizers, carriers, excipients or co-therapeutics. G-CSFR or sG-CSFR is isolated if it is detectable as a single protein band in a polyacrylamide gel by silver staining.

Isolation of cDNAs Encoding G-CSFR

The coding sequence of a mammalian G-CSFR is obtained by first isolating a cDNA sequence encoding G-CSFR from a recombinant DNA library generated using either genomic DNA or cDNA. The preferred method for constructing a cDNA library is to prepare polyadenylated mRNA obtained from a particular cell line which expresses a mammalian G-CSFR and converting the polyadenylated RNA to cDNA by reverse transcription. A particularly preferred cellular source of mRNA for construction of the cDNA library is human placental RNA.

A cDNA library will contain G-CSFR sequences which can be readily identified by screening the library with an appropriate nucleic acid probe which is capable of hybridizing with G-CSFR cDNA. Such probes can be derived from the nucleotide sequences disclosed herein. Alternatively, DNAs encoding G-CSFR proteins can also be assembled by ligation of synthetic oligonucleotide subunits to provide a complete coding sequence.

The cDNAs encoding G-CSFR of the present invention were isolated by the method of direct expression cloning. Specifically, a cDNA library was constructed by first isolating cytoplasmic mRNA from human placental tissue using standard techniques. Polyadenylated mRNA was isolated and used to prepare double-stranded cDNA. Purified cDNA fragments were then ligated into psfCAV vector DNA described in detail below in Example 2. The psfCAV vectors containing the G-CSFR cDNA fragments were transformed into *E. coli* str DH5α. Transformants were plated to provide approximately 800 colonies per plate. The resulting colonies were harvested and each pool used to prepare plasmid DNA for transfection into COS-7 cells essentially as described by Cosman et al. (*Nature 312*:768, 1984) and Luthman et al. (*Nucl. Acid Res. 11*:1295, 1983). Transformants expressing biologically active cell surface G-CSF receptors were identified by screening for the ability of G-CSFR to bind ^{125}I-G-CSF (5×10^{-10} M). Specifically, transfected COS-7 cells were incubated with medium containing ^{125}I-G-CSF, the cells washed to remove unbound labeled G-CSF, and the cell monolayers contacted with X-ray film to detect concentrations of G-CSF binding, as disclosed by Sims et al, *Science*

241:585 (1988). Transfectants detected in this manner appear as dark foci against a relatively light background.

This approach as used to screen approximately 30,000 cDNAs in pools of approximately 600 cDNAs until assay of a transfectant pool indicated positive foci for G-CSF binding. A frozen stock of bacteria from this positive pool was grown in culture and plated to provide individual colonies, which were screened until single clones were identified which are capable of directing synthesis of a surface protein with detectable G-CSF binding activity. Additional cDNA clones can be isolated from cDNA libraries of other mammalian species by cross-species hybridization of human G-CSFR cDNAs with cDNA derived from other mammalian species. For use in hybridization, DNA encoding G-CSFR may be covalently labeled with a detectable substance such as a fluorescent group, a radioactive atom or a chemiluminescent group by methods well known to those skilled in the art. Such probes could also be used for *in vitro* diagnosis of particular conditions.

Like most mammalian genes, mammalian G-CSF receptors are presumably encoded by multi-exon genes. Alternative mRNA constructs which can be attributed to different mRNA splicing events following transcription, and which share large regions of identity or similarity with the cDNAs claimed herein, are considered to be within the scope of the present invention.

Proteins and Analogs

The present invention provides isolated recombinant mammalian G-CSFR polypeptides as defined above. Isolated G-CSFR polypeptides are substantially free of other contaminating materials of natural or endogenous origin and contain less than about 1% by mass of protein contaminants residual of production processes. Such polypeptides are optionally without associated native-pattern glycosylation. Mammalian G-CSFR of the present invention includes, by way of example, primate, human, murine, canine, feline, bovine, ovine, equine and porcine G-CSFR. Derivatives of G-CSFR within the scope of the invention also include various structural forms of the primary protein which retain biological activity. Due to the presence of ionizable amino and carboxyl groups, for example, a G-CSFR protein may be in the form of acidic or basic salts, or may be in neutral form. Individual amino acid residues may also be modified by oxidation or reduction.

The primary amino acid structure may be modified by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence mutants. Covalent derivatives are prepared by linking particular functional groups to G-CSFR amino acid side chains or at the N- or C-termini. Other derivatives of G-CSFR within the scope

of this invention include covalent or aggregative conjugates of G-CSFR or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugated peptide may be a a signal (or leader) polypeptide sequence at the N-terminal region of the protein which co-translationally or post-translationally directs transfer of the protein from its site of synthesis to its site of function inside or outside of the cell membrane or wall (e.g., the yeast α-factor leader). G-CSFR protein fusions can comprise peptides added to facilitate purification or identification of G-CSFR (e.g., poly-His). The amino acid sequence of G-CSF receptor can also be linked to the peptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (DYKDDDDK) (Hopp et al., *BioITechnology* 6:1204,1988.) The latter sequence is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. This sequence is also specifically cleaved by bovine mucosal enterokinase at the residue immediately following the Asp-Lys pairing. Fusion proteins capped with this peptide may also be resistant to intracellular degradanon in *E. coli*.

G-CSFR derivatives may also be used as immunogens, reagents in receptor-based immunoassays, or as binding agents for affinity purification procedures of G-CSF or other binding ligands. G-CSFR derivatives may also be obtained by cross-linking agents, such as M-maleimidobenzoyl succinimide ester and N-hydroxysuccinimide, at cysteine and lysine residues. G-CSFR proteins may also be covalently bound through reactive side groups to various insoluble substrates, such as cyanogen bromide-activated, bisoxirane-activated, carbonyldiimidazole-activated or tosyl-activated agarose structures, or by adsorbing to polyolefin surfaces (with or without glutaraldehyde cross-linking). Once bound to a substrate, G-CSFR may be used to selectively bind (for purposes of assay or purification) anti-G-CSFR antibodies or G-CSF.

The present invention also includes G-CSFR with or without associated native-pattern glycosylation. G-CSFR expressed in yeast or mammalian expression systems, e.g., COS-7 cells, may be similar or slightly different in molecular weight and glycosylation pattern than the native molecules, depending upon the expression system. Expression of G-CSFR DNAs in bacteria such as *E. coli* provides non-glycosylated molecules. Functional mutant analogs of mammalian G-CSFR having inactivated N-glycosylation sites can be duced by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques. These analog proteins can be produced in a homogeneous, reduced-carbohydrate form in good yield using yeast expression systems. N-glycosylation sites in eukaryotic proteins are characterized by the amino acid triplet Asn-A_1-Z, where A_1 is any amino acid except Pro, and Z is Ser or Thr. In this sequence, asparagine provides a side chain amino group for covalent attachment of carbohydrate.

Such a site can be eliminated by substituting another amino acid for Asn or for residue Z, deleting Asn or Z, or inserting a non-Z amino acid between A_1 and Z, or an amino acid other than Asn between Asn and A_1.

G-CSFR derivatives may also be obtained by mutations of G-CSFR or its subunits. A G-CSFR mutant, as referred to herein, is a polypeptide homologous to G-CSFR but which has an amino acid sequence different from native G-CSFR because of a deletion, insertion or substitution.

Bioequivalent analogs of G-CSFR proteins may be constructed by, for example, making various substitutions of residues or sequences or deleting terminal or internal residues or sequences not needed for biological activity. For example, aliphatic amino acid residues, such as Ile, Val, Leu or Ala may be substituted for one another, or polar amino acid residues, such as Lys and Arg, Glu and Asp, or Gln and Asn, may be substituted for one another. Also, cysteine residues can be deleted or replaced with other amino acids to prevent formation of incorrect intramolecular disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. Generally, substitutions should be made conservatively; i.e., the most preferred substitute amino acids are those having physicochemical characteristics resembling those of the residue to be replaced. Similarly, when a deletion or insertion strategy is adopted, the potential effect of the deletion or insertion on biological activity should be considered.

Subunits of G-CSFR may be constructed by deleting terminal or internal residues or sequences. Particularly preferred subunits include those in which the transmembrane region and intracellular domain of G-CSFR are deleted or substituted with hydrophilic residues to facilitate secretion of the receptor into the cell culture medium. The resulting protein is a soluble truncated G-CSFR molecule which may retain its ability to bind G-CSF.

Mutations in nucleotide sequences constructed for expression of analog G-CSFR must, of course, preserve the reading frame phase of the coding sequences and preferably will not create complementary regions that could hybridize to produce secondary mRNA structures such as loops or hairpins which would adversely affect translation of the receptor mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the mutation *per se* be predetermined. For example, in order to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at the target codon and the expressed G-CSFR mutants screened for the desired activity.

Not all mutations in the nucleotide sequence which encodes G-CSFR will be expressed in the final product, for example, nucleotide substitutions may be made to enhance expression, primarily to avoid secondary structure loops in the transcribed mRNA

(see EPA 75,444A, incorporated herein by reference), or to provide codons that are more readily translated by the selected host, e.g., the well-known *E. coli* preference codons for *E. coli* expression.

Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene 42*:133, 1986); Bauer et al. (*Gene 37*:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); and U.S. Patent Nos. 4,518,584 and 4,737,462 disclose suitable techniques, and are incorporated by reference herein.

Expression of Recombinant G-CSFR

The present invention provides recombinant expression vectors which include synthetic or cDNA-derived DNA fragments encoding mammalian G-CSFR or bioequivalent analogs operably linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. Such regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation, as described in detail below. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated. DNA regions are operably linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of secretory leaders, contiguous and in reading frame.

DNA sequences encoding mammalian G-CSF receptors which are to be expressed in a microorganism will preferably contain no introns that could prematurely terminate transcription of DNA into mRNA; however, premature termination of transcription may be desirable, for example, where it would result in mutants having advantageous C-terminal

truncations, for example, deletion of a transmembrane region to yield a soluble receptor not bound to the cell membrane. Due to code degeneracy, there can be considerable variation in nucleotide sequences encoding the same amino acid sequence. Other embodiments include sequences capable of hybridizing to the sequences of the provided cDNA under moderately stringent conditions (50°C, 2 X SSC) and other sequences hybridizing or degenerate to those which encode biologically active G-CSF receptor polypeptides.

Transformed host cells are cells which have been transformed or transfected with G-CSFR vectors constructed using recombinant DNA techniques. Transformed host cells ordinarily express G-CSFR, but host cells transformed for purposes of cloning or amplifying G-CSFR DNA do not need to express G-CSFR. Expressed G-CSFR will be deposited in the cell membrane or secreted into the culture supernatant, depending on the G-CSFR DNA selected. Suitable host cells for expression of mammalian G-CSFR include prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed to produce mammalian G-CSFR using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al. (*Cloning Vectors: A Laboratory Manual*, Elsevier, New York, 1985), the relevant disclosure of which is hereby incorporated by reference.

Prokaryotic expression hosts may be used for expression of G-CSFR that do not require extensive proteolytic and disulfide processing. Prokaryotic expression vectors generally comprise one or more phenotypic selectable markers, for example a gene encoding proteins conferring antibiotic resistance or supplying an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli, Bacillus subtilis, Salmonella typhimurium*, and various species within the genera *Pseudomonas, Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA) and pCAV/NOT (ATCC Accession No. 68014. These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed. *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species

(Bolivar et al., *Gene* 2:95, 1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells.

Promoters commonly used in recombinant microbial expression vectors include the β-lactamase (penicillinase) and lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), the tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EPA 36,776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful bacterial expression system employs the phage λ P_L promoter and cI857ts thermolabile repressor. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2, resident in *E. coli* strain JMB9 (ATCC 37092) and pPLc28, resident in *E. coli* RR1 (ATCC 53082).

Recombinant G-CSFR proteins may also be expressed in yeast hosts, preferably from the *Saccharomyces* species, such as *S. cerevisiae*. Yeast of other genera, such as *Pichia* or *Kluyveromyces* may also be employed. Yeast vectors will generally contain an origin of replication from the 2μ yeast plasmid or an autonomously replicating sequence (ARS), promoter, DNA encoding G-CSFR, sequences for polyadenylation and transcription termination and a selection gene. Preferably, yeast vectors will include an origin of replication and selectable marker permitting transformation of both yeast and *E. coli*, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* trp1 gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, and a promoter derived from a highly expressed yeast gene to induce transcription of a structural sequence downstream. The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoter sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPA 73,657.

Preferred yeast vectors can be assembled using DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) and yeast DNA sequences including a glucose-repressible ADH2 promoter and α-factor secretion leader. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982)

and Beier et al. (*Nature 300*:724, 1982). The yeast α-factor leader, which directs secretion of heterologous proteins, can be inserted between the promoter and the structural gene to be expressed. *See, e.g.*, Kurjan et al., *Cell 30*:933, 1982; and Bitter et al., *Proc. Natl. Acad. Sci. USA 81*:5330, 1984. The leader sequence may be modified to contain, near its 3' end, one or more useful restriction sites to facilitate fusion of the leader sequence to foreign genes.

Suitable yeast transformation protocols are known to those of skill in the art; an exemplary technique is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA 75*:1929, 1978, selecting for Trp^+ transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Host strains transformed by vectors comprising the ADH2 promoter may be grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs upon exhaustion of medium glucose. Crude yeast supernatants are harvested by filtration and held at 4°C prior to further purification.

Various mammalian or insect cell culture systems can be employed to express recombinant protein. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988). Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman (*Cell 23*:175, 1981), and other cell lines capable of expressing an appropriate vector including, for example, L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors may comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. The early and late promoters are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature 273*:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending

from the *Hind* III site toward the *BglI* site located in the viral origin of replication is included. Further, mammalian genomic G-CSFR promoter, control and/or signal sequences may be utilized, provided such control sequences are compatible with the host cell chosen. Additional details regarding the use of a mammalian high expression vector to produce a recombinant mammalian G-CSF receptor are provided in Example 2 below. Exemplary vectors can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol. 3*:280, 1983).

A useful system for stable high level expression of mammalian receptor cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol. 23*:935, 1986).

A particularly preferred eukaryotic vector for expression of G-CSFR DNA is disclosed below in Example 2. This vector, referred to as pCAV/NOT, was derived from the mammalian high expression vector pDC201 and contains regulatory sequences from SV40, adenovirus-2, and human cytomegalovirus.

Purified mammalian G-CSF receptors or analogs are prepared by culturing suitable host/vector systems to express the recombinant translation products of the DNAs of the present invention, which are then purified from culture media or cell extracts.

For example, supernatants from systems which secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. For example, a suitable affinity matrix can comprise a G-CSF or lectin or antibody molecule bound to a suitable support. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred.

Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a G-CSFR composition. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

Recombinant protein produced in bacterial culture is usually isolated by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells

employed in expression of recombinant mammalian G-CSFR can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Fermentation of yeast which express mammalian G-CSFR as a secreted protein greatly simplifies purification. Secreted recombinant protein resulting from a large-scale fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). This reference describes two sequential, reversed-phase HPLC steps for purification of recombinant human GM-CSF on a preparative HPLC column.

Human G-CSFR synthesized in recombinant culture is characterized by the presence of non-human cell components, including proteins, in amounts and of a character which depend upon the purification steps taken to recover human G-CSFR from the culture. These components ordinarily will be of yeast, prokaryotic or non-human higher eukaryotic origin and preferably are present in innocuous contaminant quantities, on the order of less than about 1 percent by weight. Further, recombinant cell culture enables the production of G-CSFR free of proteins which may be normally associated with G-CSFR as it is found in nature in its species of origin, e.g. in cells, cell exudates or body fluids.

G-CSFR compositions are prepared for administration by mixing G-CSFR having the desired degree of purity with physiologically acceptable carriers. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the G-CSFR with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients.

G-CSFR compositions may be used to attenuate G-CSF-mediated immune responses. To achieve this result, a therapeutically effective quantity of a G-CSFR composition is administered to a mammal, preferably a human, in association with a pharmaceutical carrier or diluent.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

Example 1

Binding Assays

A. *Radiolabeling of G-CSF.* Recombinant human G-CSF, in the form of a fusion protein containing a hydrophilic octapeptide at the N-terminus, was expressed in yeast as a

secreted protein and purified by affinity chromatography as described by Hopp et al., *Bio/Technology* 6:1204, 1988. The protein was radiolabeled using the commercially available solid phase agent, IODO-GEN (Pierce). In this procedure, 5 µg of IODO-GEN were plated at the bottom of a 10 X 75 mm glass tube and incubated for 20 minutes at 4°C with 75 µl of 0.1 M sodium phosphate, pH 7.4 and 20 µl (2 mCi) Na ^{125}I. This solution was then transferred to a second glass tube containing 5 µg G-CSF in 45 µl PBS for 20 minutes at 4°C. The reaction mixture was fractionated by gel filtration on a 2 ml bed volume of Sephadex G-25 (Sigma) equilibrated in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2.5% (w/v) bovine serum albumin (BSA), 0.2% (w/v) sodium azide and 20 mM Hepes pH 7.4 (binding medium). The final pool of ^{125}I-G-CSF was diluted to a working stock solution of 1 x 10^{-7} M in binding medium and stored for up to one month at 4°C without detectable loss of receptor binding activity. The specific activity is routinely 1 x 10^{16} cpm/mmole G-CSF. Radiolabeled G-CSF is used as described below to assay for G-CSF receptors.

B. *Membrane Binding Assays.* Human placental membranes were incubated at 4°C for 2 hr with ^{125}I-G-CSF in binding medium, 0.1% bacitracin, 0.02% aprotinin, and 0.4% BSA in a total volume of 1.2 ml. Control tubes containing in addition a 100 x molar excess of unlabeled G-CSF were also included to determine non-specific binding. The reaction mixture was then centrifuged at 15,000x g in a microfuge for 5 minutes. Supernatants were discarded, the surface of the membrane pellets carefully rinsed with ice-cold binding medium, and the radioactivity counted on a gamma counter. Using this assay, it was determined that the G-CSFR present in the COS cell supernatants of Example 2 had a K_a of about 1 x 10^9 M^{-1} and a molecular weight of about 35 kDa.

C. *Solid Phase Binding Assays.* The ability of G-CSFR to be stably adsorbed to nitrocellulose from detergent extracts of human cells yet retain G-CSF-binding activity provided a means of detecting G-CSFR. Cells extracts were prepared by mixing a cell pellet with a 2X volume of PBS containing 1% Triton X-100 and a cocktail of protease inhibitors (2 mM phenylmethyl sulfonyl fluoride, 10 µM pepstatin, 10 µM leupeptin, 2 mM o-phenanthroline and 2 mM EGTA) by vigorous vortexing. The mixture was incubated on ice for 30 minutes after which it was centrifuged at 12,000x g for 15 minutes at 8°C to remove nuclei and other debris. Two microliter aliquots of cell extracts were placed on dry BA85/21 nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and allowed to dry. The membranes were incubated in tissue culture dishes for 30 min. rocks in Tris (0.05 M) buffered saline (0.15 M) pH 7.5 containing 3% w/v BSA to block nonspecific binding sites. The membrane was then covered with 0.3 nM ^{125}I-G-CSF in PBS + 3% BSA and incubated for 2 hr at 4°C with shaking. At the end of this time, the membranes were washed 3 times in PBS, dried and placed on Kodak X-Omat AR film for 18 hr at -70°C.

This assay was performed to detect the presence of G-CSFR in various cells lines and tissue sources.

D. *Binding Assay for Soluble G-CSFR.* Soluble G-CSFR present in COS-7 cell supernatants are measured by inhibition of ^{125}I-CSF binding to a G-CSF-dependent cell line, or any other human cell or cell line expressing G-CSF receptors, such as as human placental cell. Supernatants are harvested from COS-7 cells 3 days after transfection, concentrated 10-fold, and preincubated with ^{125}I-G-CSF for 1 hour at 37°C. Appropriate G-CSF-receptor-bearing cells are added to a final volume of 150 ul, incubated for an additional 30 minutes at 37°C, and assayed and analyzed as described by Park et al., *J. Biol. Chem. 261*:4177 (1986).

Example 2

Isolation of Human G-CSF R cDNAs by Direct Expression of Active Protein in COS-7 Cells

A tissue source for G-CSFR was selected by screening various human cell lines and tissues for expression of G-CSFR based on their ability to bind ^{125}I-labeled G-CSF, prepared as described above in Example 1A. Human placental membranes were found to express a reasonable number of receptors. Equilibrium binding studies were performed according to Example 1B and showed that the membrane exhibited biphasic binding of ^{125}I-G-CSF with high affinity sites ($K_a = 4 \times 10^{19}$ M^{-1}) of 0.4 pmoles receptor/mg protein.

An unsized cDNA library was constructed by reverse transcription of polyadenylated mRNA isolated from total RNA extracted from the human placental tissue (Ausubel et al., eds., *Current Protocols in Molecular Biology*, Vol. 1, 1987). The cells were harvested by lysing the tissue cells in a guanidinium isothiocyanate solution and total RNA was isolated using standard techniques as described by Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1982.

Polyadenylated RNA was isolated by oligo dT cellulose chromatography and double-stranded cDNA was prepared by a method similar to that of Gubler and Hoffman, *Gene 25*:263, 1983. Briefly, the polyadenylated RNA was converted to an RNA-cDNA hybrid with reverse transcriptase using oligo dT as a primer. The RNA-cDNA hybrid was then converted into double-stranded cDNA using RNAase H in combination with DNA polymerase I. The resulting double stranded cDNA was blunt-ended with T4 DNA polymerase. *Bgl*II adaptors were ligated to the 5' ends of the resulting blunt-ended cDNA as described by Haymerle, et al., *Nuclear Acids Research, 14*: 8615, 1986. The non-ligated adaptors were removed by gel filtration chromatography at 68°C, leaving 24 nucleotide non-self-complementary overhangs on the cDNA. The same

procedure was used to convert the 5' *BglII* ends of the mammalian expression vector psfCAV to 24 nucleotide overhangs complementary to those added to the cDNA. Optimal proportions of adaptored vector and cDNA were ligated in the presence of T4 polynucleotide kinase. Dialyzed ligation mixtures were electroporated into *E. coli* strain DH5α and transformants selected on ampicillin plates.

The resulting cDNAs were ligated into the eukaryotic expression vector psfCAV, which was designed to express cDNA sequences inserted at its multiple cloning site when transfected into mammalian cells. psfCAV was assembled from pDC201 (a derivative of pMLSV, previously described by Cosman et al., *Nature 312*: 768, 1984), SV40 and cytomegalovirus DNA and comprises, in sequence with the direction of transcription from the origin of replication: (1) SV40 sequences from coordinates 5171-5270 containing the origin of replication, enhancer sequences and early and late promoters; (2) cytomegalovirus sequences containing the promoter and enhancer regions (nucleotides 671 to +63 from the sequence published by Boechart et al. (*Cell 41*:521, 1985); (3) adenovirus-2 sequences from coordinates 5779-6079 containing sequences for the motor late promoter and the first exon of the tripartite leader (TPL), coordinates 7101-7172 and 9634-9693 containing the second exon and part of the third exon of the TPL and a multiple cloning site (MCS) containing sites for XhoI, KpnI, SmaI and*BglI*; (4) SV40 sequences from coordinates 4127-4100 and 2770-2533 containing the polyadenylation and termination signals for early transcription; (5) with adenovirus sequences from coordinates 10532-11156 of the virus-associated RNA genes VAI and VAII of pDC201; and (6) pBR322 sequences from coordinates 4363-2486 and 1094-375 containing the ampicillin resistance gene and origin of replication.

The resulting human placental cDNA library in sfCAV was used to transform *E. coli* strain DH5α, and recombinants were plated to provide approximately 500-600 colonies per plate and sufficient plates to provide approximately 30,000 total colonies per screen. Colonies were scraped from each plate, pooled, and plasmid DNA prepared from each pool. The pooled DNA was then used to transfect a sub-confluent layer of monkey COS-7 cells using DEAE-dextran followed by chloroquine treatment, as described by Luthman et al., *Nucl. Acids Res. 11*:1295 (1983) and McCutchan et al., *J. Natl. Cancer Inst. 41*:351 (1986). The cells were then grown in culture for three days to permit transient expression of the inserted sequences. After three days, cell culture supernatants were discarded and the cell monolayers in each plate assayed for G-CSF binding as follows. Three ml of binding medium containing 1.2×10^{-11} M ^{125}I-labeled flag-G-CSF was added to each plate and the plates incubated at 4°C for 120 minutes. This medium was then discarded, and each plate was washed once with cold binding medium (containing no labeled G-CSF) and twice with cold PBS. The edges of each plate were then broken off, leaving a flat disk

which was contacted with X-ray film for 72 hours at -70°C using an intensifying screen. G-CSF binding activity was visualized on the exposed films as a dark spot against a relatively uniform background.

After approximately 30,000 recombinants from the library had been screened in this manner, nine transfectant pools were observed to provide G-CSF binding foci which were clearly apparent against the background exposure.

A frozen stock of bacteria from the positive pool was then used to obtain plates of approximately 60 colonies. Replicas of these plates were made on nitrocellulose filters, and the plates were then scraped and plasmid DNA prepared and transfected as described above to identify a positive plate. Bacteria from individual colonies from the nitrocellulose replica of this plate were grown in 0.2 ml cultures, which were used to obtain plasmid DNA. The plasmid DNA was then transfected into COS-7 cells as described above. In this manner, a single clone, clone D-7, was isolated which was capable of inducing expression of G-CSFR in COS cells. A glycerol stock of bacteria transformed with this G-CSFR cDNA clone in the expression vector pCAV/NOT (or pDC302) has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA, under accession number 68102.

An additional cDNA clone encoding G-CSFR was isolated from the same placental library. Recombinants from the placental cDNA library were plated on *E. coli* strain DH5α and transformants selected on ampicillin plates. The transformants were screened by plaque hybridization techniques under conditions of high stringency (63°C, 0.2X SSC) using a ^{32}P-labeled probe made from the human G-CSFR clone D-7. A hybridizing clone (clone 25-1) was isolated which is identical to clone D-7, except that it contains an intron insertion after nucleotide 2411, adding nucleotides 2412-2832 of Figure 6 and resulting in a change in reading frame and a corresponding change in amino acid sequence. The 3' nucleotide sequence and predicted C-terminal amino acid sequence of clone 25-1 are set forth in Figure 6.

Example 3

Construction of cDNAs Encoding Soluble Human G-CSFR

Soluble human G-CSFR was cloned into the mammalian expression vector pDC302, described above, utilizing the polymerase chain reaction (PCR) technique. The following primers were used:

5' End Primer
5'-GGTACCATGGCAAGGCTGGGAAAC
Asp718 site/Initiation Codon

3' End Primer
5'-TCTAGAACTCAGCCTCGATGTG
BglII/Termination Codon

The PCT product thus contains Asp718 and BglII restriction sites at the 5' and 3' termini, respectively. These restriction sites are used to clone into pDC302. The 3' sequence is antisense relative to sequence disclosed in Figures 2-5. The template for the PCR reaction is clone 25-1, described above, which contains the G-CSFR. The DNA sequences encoding the G-CSFR are then amplified by PCR, substantially as described by Innis et al., eds., *PCR Protocols: A Guide to Methods and Applications* (Academic Press, 1990). The resulting amplified clone was then isolated and ligated into pDC302 and expressed in monkey COS-7 cells as described above.

Example 4
Preparation of Monoclonal Antibodies to G-CSFR

Preparations of purified recombinant G-CSFR, for example, human G-CSFR, or transfected COS cells expressing high levels of G-CSFR are employed to generate monoclonal antibodies against G-CSFR using conventional techniques, for example, those disclosed in U.S. Patent 4,411,993. Such antibodies are likely to be useful in interfering with G-CSF binding to G-CSF receptors, for example, in ameliorating toxic or other undesired effects of G-CSF, or as components of diagnostic or research assays for G-CSF or soluble G-CSF receptor.

To immunize mice, G-CSFR immunogen is emulsified in complete Freund's adjuvant and injected in amounts ranging from 10-100 µg subcutaneously into Balb/c mice. Ten to twelve days later, the immunized animals are boosted with additional immunogen emulsified in incomplete Freund's adjuvant and periodically boosted thereafter on a weekly to biweekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich) or ELISA (enzyme-linked immunosorbent assay). Other assay procedures are also suitable. Following detection of an appropriate antibody titer, positive animals are given an intravenous injection of antigen in saline. Three to four days later, the animals are sacrificed, splenocytes harvested, and fused to the murine myeloma cell line NS1. Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates in a HAT selective medium (hypoxanthine, aminopterin, and thymidine) to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

Hybridoma clones thus generated can be screened by ELISA for reactivity with G-CSFR, for example, by adaptations of the techniques disclosed by Engvall et al., *Immunochem.* 8:871 (1971) and in U.S. Patent 4,703,004. Positive clones are then injected into the peritoneal cavities of syngeneic Balb/c mice to produce ascites containing high concentrations (>1 mg/ml) of anti-G-CSFR monoclonal antibody. The resulting monoclonal antibody can be purified by ammonium sulfate precipitation followed by gel exclusion chromatography, and/or affinity chromatography based on binding of antibody to Protein A of *Staphylococcus aureus*.

CLAIMS

1. An isolated DNA sequence encoding a biologically active mammalian G-CSF receptor (G-CSFR) protein.

2. A DNA sequence according to claim 1, selected from the group consisting of:

(a) cDNA clones having a nucleotide sequence derived from the coding region of a native mammalian G-CSFR gene;

(b) DNA sequences capable of hybridization to the clones of (a) under moderately stringent conditions (50°C, 2 x SSC) and which encode biologically active G-CSFR molecules; and

(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode biologically active G-CSFR molecules.

3. An isolated DNA sequence according to claim 1, encoding a soluble biologically active mammalian G-CSFR.

4. A recombinant expression vector comprising a DNA sequence according to claim 1.

5. A recombinant expression vector comprising a DNA sequence according to claim 2.

6. A recombinant expression vector comprising a DNA sequence according to claim 3.

7. A process for preparing a mammalian G-CSF receptor or an analog thereof, comprising culturing a suitable host cell comprising a vector according to claim 4 under conditions promoting expression.

8. A purified biologically active mammalian G-CSF receptor composition.

9. A purified biologically active mammalian G-CSF receptor composition according to claim 8, consisting essentially of human G-CSF receptor.

10. A composition for regulating immune or inflammatory responses in a mammal, comprising an effective amount of a mammalian G-CSF receptor protein composition according to claim 8, and a suitable diluent or carrier.

11. A method for regulating immune responses in a mammal, comprising administering an effective amount of a composition according to claim 10.

12. An assay method for detection of G-CSF or G-CSF receptor molecules or the interaction thereof, comprising use of a protein composition according to claim 8.

13. Antibodies immunoreactive with mammalian G-CSF receptors.

FIGURE 1

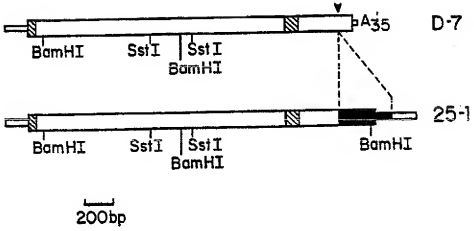
pA35
D-7
BamHI
SstI
SstI
BamHI
25-1
BamHI
SstI
SstI
BamHI
BamHI
200bp

FIG. 2

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                                TG GAC TGC AGC TGG TTT CAG GAA CTT CTC TTG    32

ACG AGA AGA GAG ACC AAG GAG GCC AAG CAG GGG CTG GGC CAG AGG TGC              80

CAA CAT GGG GAA ACT GAG GCT CGG CTC GGA AAG GTG AAG TAA CTT GTC              128

CAA GAT CAC AAA GCT GGT GAA CAT CAA GTT GGT GCT ATG GCA AGG CTG              176
                                                Met Ala Arg Leu
                                               -24

GGA AAC TGC AGC CTG ACT TGG GCT GCC CTG ATC ATC CTG CTG CTC CCC              224
Gly Asn Cys Ser Leu Thr Trp Ala Ala Leu Ile Ile Leu Leu Leu Pro
-20                 -15                 -10                  -5

GGA AGT CTG GAG GAG TGC GGG CAC ATC AGT GTC TCA GCC CCC ATC GTC              272
Gly Ser Leu Glu Glu Cys Gly His Ile Ser Val Ser Ala Pro Ile Val
                 1               5                  10

CAC CTG GGG GAT CCC ATC ACA GCC TCC TGC ATC ATC AAG CAG AAC TGC              320
His Leu Gly Asp Pro Ile Thr Ala Ser Cys Ile Ile Lys Gln Asn Cys
          15                  20                  25

AGC CAT CTG GAC CCG GAG CCA CAG ATT CTG TGG AGA CTG GGA GCA GAG              368
Ser His Leu Asp Pro Glu Pro Gln Ile Leu Trp Arg Leu Gly Ala Glu
     30                  35                  40

CTT CAG CCC GGG GGC AGG CAG CAG CGT CTG TCT GAT GGG ACC CAG GAA              416
Leu Gln Pro Gly Gly Arg Gln Gln Arg Leu Ser Asp Gly Thr Gln Glu
 45                  50                  55                  60

TCT ATC ATC ACC CTG CCC CAC CTC AAC CAC ACT CAG GCC TTT CTC TCC              464
Ser Ile Ile Thr Leu Pro His Leu Asn His Thr Gln Ala Phe Leu Ser
                 65                  70                  75

TGC TGC CTG AAC TGG GGC AAC AGC CTG CAG ATC CTG GAC CAG GTT GAG              512
Cys Cys Leu Asn Trp Gly Asn Ser Leu Gln Ile Leu Asp Gln Val Glu
          80                  85                  90

CTG CGC GCA GGC TAC CCT CCA GCC ATA CCC CAC AAC CTC TCC TGC CTC              560
Leu Arg Ala Gly Tyr Pro Pro Ala Ile Pro His Asn Leu Ser Cys Leu
     95                  100                 105

ATG AAC CTC ACA ACC AGC AGC CTC ATC TGC CAG TGG GAG CCA GGA CCT              608
Met Asn Leu Thr Thr Ser Ser Leu Ile Cys Gln Trp Glu Pro Gly Pro
    110                 115                 120

GAG ACC CAC CTA CCC ACC AGC TTC ACT CTG AAG AGT TTC AAG AGC CGG              656
Glu Thr His Leu Pro Thr Ser Phe Thr Leu Lys Ser Phe Lys Ser Arg
125                 130                 135                 140

GGC AAC TGT CAG ACC CAA GGG GAC TCC ATC CTG GAC TGC GTG CCC AAG              704
Gly Asn Cys Gln Thr Gln Gly Asp Ser Ile Leu Asp Cys Val Pro Lys
                145                 150                 155

GAC GGG CAG AGC CAC TGC TGC ATC CCA CGC AAA CAC CTG CTG TTG TAC              752
Asp Gly Gln Ser His Cys Cys Ile Pro Arg Lys His Leu Leu Leu Tyr
            160                 165                 170
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FIG. 3

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CAG AAT ATG GGC ATC TGG GTG CAG GCA GAG AAT GCG CTG GGG ACC AGC  800
Gln Asn Met Gly Ile Trp Val Gln Ala Glu Asn Ala Leu Gly Thr Ser
        175                 180                 185

ATG TCC CCA CAA CTG TGT CTT GAT CCC ATG GAT GTT GTG AAA CTG GAG  848
Met Ser Pro Gln Leu Cys Leu Asp Pro Met Asp Val Val Lys Leu Glu
    190                 195                 200

CCC CCC ATG CTG CGG ACC ATG GAC CCC AGC CCT GAA GCG GCC CCT CCC  896
Pro Pro Met Leu Arg Thr Met Asp Pro Ser Pro Glu Ala Ala Pro Pro
205                 210                 215                 220

CAG GCA GGC TGC CTA CAG CTG TGC TGG GAG CCA TGG CAG CCA GGC CTG  944
Gln Ala Gly Cys Leu Gln Leu Cys Trp Glu Pro Trp Gln Pro Gly Leu
                225                 230                 235

CAC ATA AAT CAG AAG TGT GAG CTG CGC CAC AAG CCG CAG CGT GGA GAA  992
His Ile Asn Gln Lys Cys Glu Leu Arg His Lys Pro Gln Arg Gly Glu
            240                 245                 250

GCC AGC TGG GCA CTG GTG GGC CCC CTC CCC TTG GAG GCC CTT CAG TAT 1040
Ala Ser Trp Ala Leu Val Gly Pro Leu Pro Leu Glu Ala Leu Gln Tyr
        255                 260                 265

GAG CTC TGC GGG CTC CTC CCA GCC ACG GCC TAC ACC CTG CAG ATA CGC 1088
Glu Leu Cys Gly Leu Leu Pro Ala Thr Ala Tyr Thr Leu Gln Ile Arg
    270                 275                 280

TGC ATC CGC TGG CCC CTG CCT GGC CAC TGG AGC GAC TGG AGC CCC AGC 1136
Cys Ile Arg Trp Pro Leu Pro Gly His Trp Ser Asp Trp Ser Pro Ser
285                 290                 295                 300

CTG GAG CTG AGA ACT ACC GAA CGG GCC CCC ACT GTC AGA CTG GAC ACA 1184
Leu Glu Leu Arg Thr Thr Glu Arg Ala Pro Thr Val Arg Leu Asp Thr
                305                 310                 315

TGG TGG CGG CAG AGG CAG CTG GAC CCC AGG ACA GTG CAG CTG TTC TGG 1232
Trp Trp Arg Gln Arg Gln Leu Asp Pro Arg Thr Val Gln Leu Phe Trp
            320                 325                 330

AAG CCA GTG CCC CTG GAG GAA GAC ACC GGA CGG ATC CAA GGT TAT GTG 1280
Lys Pro Val Pro Leu Glu Glu Asp Ser Gly Arg Ile Gln Gly Tyr Val
        335                 340                 345

GTT TCT TGG AGA CCC TCA GGC CAG GCT GGG GCC ATC CTG CCC CTC TGC 1328
Val Ser Trp Arg Pro Ser Gly Gln Ala Gly Ala Ile Leu Pro Leu Cys
    350                 355                 360

AAC ACC ACA GAG CTC AGC TGC ACC TTC CAC CTG CCT TCA GAA GCC CAG 1376
Asn Thr Thr Glu Leu Ser Cys Thr Phe His Leu Pro Ser Glu Ala Gln
365                 370                 375                 380

GAG GTG GCC CTT GTG GCC TAT AAC TCA GCC GGG ACC TCT CGC CCC ACC 1424
Glu Val Ala Leu Val Ala Tyr Asn Ser Ala Gly Thr Ser Arg Pro Thr
                385                 390                 395
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FIG. 4

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CCG GTG GTC TTC TCA GAA AGC AGA GGC CCA GCT CTG ACC AGA CTC CAT 1472
Pro Val Val Phe Ser Glu Ser Arg Gly Pro Ala Leu Thr Arg Leu His
            400                 405                 410

GCC ATG GCC CGA GAC CCT CAC AGC CTC TGG GTA GGC TGG GAG CCC CCC 1520
Ala Met Ala Arg Asp Pro His Ser Leu Trp Val Gly Trp Glu Pro Pro
        415                 420                 425

AAT CCA TGG CCT CAG GGC TAT GTG ATT GAG TGG GGC CTG GGC CCC CCC 1568
Asn Pro Trp Pro Gln Gly Tyr Val Ile Glu Trp Gly Leu Gly Pro Pro
    430                 435                 440

AGC GCG AGC AAT AGC AAC AAG ACC TGG AGG ATG GAA CAG AAT GGG AGA 1616
Ser Ala Ser Asn Ser Asn Lys Thr Trp Arg Met Glu Gln Asn Gly Arg
445                 450                 455                 460

GCC ACG GGG TTT CTG CTG AAG GAG AAC ATC AGG CCC TTT CAG CTC TAT 1664
Ala Thr Gly Phe Leu Leu Lys Glu Asn Ile Arg Pro Phe Gln Leu Tyr
                465                 470                 475

GAG ATC ATC GTG ACT CCC TTG TAC CAG GAC ACC ATG GGA CCC TCC CAG 1712
Glu Ile Ile Val Thr Pro Leu Tyr Gln Asp Thr Met Gly Pro Ser Gln
            480                 485                 490

CAT GTC TAT GCC TAC TCT CAA GAA ATG GCT CCC TCC CAT GCC CCA GAG 1760
His Val Tyr Ala Tyr Ser Gln Glu Met Ala Pro Ser His Ala Pro Glu
        495                 500                 505

CTG CAT CTA AAG CAC ATT GGC AAG ACC TGG GCA CAG CTG GAG TGG GTG 1808
Leu His Leu Lys His Ile Gly Lys Thr Trp Ala Gln Leu Glu Trp Val
    510                 515                 520

CCT GAG CCC CCT GAG CTG GGG AAG AGC CCC CTT ACC CAC TAC ACC ATC 1856
Pro Glu Pro Pro Glu Leu Gly Lys Ser Pro Leu Thr His Tyr Thr Ile
525                 530                 535                 540

TTC TGG ACC AAC GCT CAG AAC CAG TCC TTC TCC GCC ATC CTG AAT GCC 1904
Phe Trp Thr Asn Ala Gln Asn Gln Ser Phe Ser Ala Ile Leu Asn Ala
                545                 550                 555

TCC TCC CGT GGC TTT GTC CTC CAT GGC CTG GAG CCC GCC AGT CTG TAT 1952
Ser Ser Arg Gly Phe Val Leu His Gly Leu Glu Pro Ala Ser Leu Tyr
            560                 565                 570

CAC ATC CAC CTC ATG GCT GCC AGC CAG GCT GGG GCC ACC AAC AGT ACA 2000
His Ile His Leu Met Ala Ala Ser Gln Ala Gly Ala Thr Asn Ser Thr
        575                 580                 585

GTC CTC ACC CTG ATG ACC TTG ACC CCA GAG GGG TCG GAG CTA CAC ATC 2048
Val Leu Thr Leu Met Thr Leu Thr Pro Glu Gly Ser Glu Leu His Ile
    590                 595                 600

ATC CTG GGC CTG TTC GGC CTC CTG CTG TTG CTC ACC TGC CTC TGT GGA 2096
Ile Leu Gly Leu Phe Gly Leu Leu Leu Leu Leu Thr Cys Leu Cys Gly
605                 610                 615                 620
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FIG. 5

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ACT GCC TGG CTC TGT TGC AGC CCC AAC AGG AAG AAT CCC CTC TGG CCA 2144
Thr Ala Trp Leu Cys Cys Ser Pro Asn Arg Lys Asn Pro Leu Trp Pro
                625                 630                 635

AGT GTC CCA GAC CCA GCT CAC AGC AGC CTG GGC TCC TGG GTG CCC ACA 2192
Ser Val Pro Asp Pro Ala His Ser Ser Leu Gly Ser Trp Val Pro Thr
            640                 645                 650

ATC ATG GAG GAG GAT GCC TTC CAG CTG CCC GGC CTT GGC ACG CCA CCC 2240
Ile Met Glu Glu Asp Ala Phe Gln Leu Pro Gly Leu Gly Thr Pro Pro
        655                 660                 665

ATC ACC AAG CTC ACA GTG CTG GAG GAG GAT GAA AAG AAG CCG GTG CCC 2288
Ile Thr Lys Leu Thr Val Leu Glu Glu Asp Glu Lys Lys Pro Val Pro
    670                 675                 680

TGG GAG TCC CAT AAC AGC TCA GAG ACC TGT GGC CTC CCC ACT CTG GTC 2336
Trp Glu Ser His Asn Ser Ser Glu Thr Cys Gly Leu Pro Thr Leu Val
685                 690                 695                 700

CAG ACC TAT GTG CTC CAG GGG GAC CCA AGA GCA GTT TCC ACC CAG CCC 2384
Gln Thr Tyr Val Leu Gln Gly Asp Pro Arg Ala Val Ser Thr Gln Pro
                705                 710                 715

                                    ↓
CAA TCC CAG TCT GGC ACC AGC GAT CAG GCT GGG CCT CCC AGG CGA TCT 2432
Gln Ser Gln Ser Gly Thr Ser Asp Gln Ala Gly Pro Pro Arg Arg Ser
            720                 725                 730

GCA TAC TTT AAG GAC CAG ATC ATG CTC CAT CCA GCC CCA CCC AAT GGC 2480
Ala Tyr Phe Lys Asp Gln Ile Met Leu His Pro Ala Pro Pro Asn Gly
        735                 740                 745

CTT TTG TGC TTG TTT CCT ATA ACT TCA GTA TTG TAA ACTAGTTTTT      2526
Leu Leu Cys Leu Phe Pro Ile Thr Ser Val Leu
    750                 755

GGTTTGCAAA AAAAAAAAA                                           2546
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FIG. 6

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                                        CAG GTC CTT TAT GGG CAG CTG CTG  2432
                                        Gln Val Leu Tyr Gly Gln Leu Leu
                                            750                 755

GGC AGC CCC ACA AGC CCA GGG CCA GGG CAC TAT CTC CGT GTG ACT CCA  2480
Gly Ser Pro Thr Ser Pro Gly Pro Gly His Tyr Leu Arg Val Thr Pro
            760                 765                 770

CTC AGC CCC TCT TGG CGG GCC TCA CCC CCA GCC CCA AGT CCT ATG AGA  2528
Leu Ser Pro Ser Trp Arg Ala Ser Pro Pro Ala Pro Ser Pro Met Arg
        775                 780                 785

ACC TCT GGT TCC AGG CCA GCC CCT TGG GGA CCC TGG TAA CCC CAG CCC  2576
Thr Ser Gly Ser Arg Pro Ala Pro Trp Gly Pro Trp
    790                 795                 800

CAA GCC AGG AGG ACG ACT GTG TCT TTG GGC CAC TGC TCA ACT TCC CCC  2624

TCC TGC AGG GGA TCC GGG TCC ATG GGA TGG AGG CGC TGG GGA GCT TCT  2672

AGG GCT TCC TGG GGT TCC CTT CTT GGG CCT GCC TTT TAA AGG CCT GAG  2720

CTA GCT GGA GAA GAG GGG AGG GTC CAT AAG CCC ATG ACT AAA AAC TAC  2768

CCC AGC CCA GGC TCT CAC CAT CTC CAG TCA CCA GCA TCT CCC TCT CCT  2816

CCC AAT CTC CAT AGG CTG GGC CTC CCA GGC GAT CTG CAT ACT TTA AGG  2864

ACC AGA TCA TGC TCC ATC CAG CCC CAC CCA ATG GCC TTT TGT GCT TGT  2912

TTC CTA TAA CTT CAG TATT                                        2931
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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/05434

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/12, C 12 F 21/02, C 07 K 13/00, A 61 K 37/02, C 12 P 21/08, G 01 N 33/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
IPC[5]	C 12 N, C 12 P, C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	Cell, vol. 61, 20 April 1990, Cell Press, (Cambridge, MA, US), R. Fukunaga et al.: "Expression cloning of a receptor for murine granulocyte colony-stimulating factor", pages 341-350 see the whole article --	1,2,4-8
A	The Journal of Biological Chemistry, vol. 261, no. 26, 15 September 1986, The American Society of Biological Chemists, Inc., N.A. Nicola et al.: "Identification of distinct receptors for two hemopoietic growth factors (granulocyte colony-stimulating factor and multipotential colony-stimulating factor) by chemical cross-linking", pages 12384-12389 see the whole article --	1

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23rd January 1991	04. 03. 91
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	Alfredo Pelm

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION C .TINUED FROM THE SECOND SHEET

A	Blood, vol. 74, no. 1, July 1989, Grune & Straton, Inc., L.S. Park et al.: "Interleukin-3, GM-CSF, and G-CSF receptor expression on cell lines and primary leukemia cells: Receptor heterogenesity and relationship to growth factor responsiveness", pages 56-65 see the whole article ----------	1

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 1-1, because they relate to subject matter not required to be searched by this Authority, namely:

claims not searched, see rule 39.1 IV PCT.
Methods for treat ment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.